Effect of uneven application of azadirachtin on reproductive and anti-feedant behaviour of *Rhyzopertha dominica* (Coleoptera: Bostrichidae)

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Abstract: The effects of uneven application of an azadirachtin-enriched neem extract to wheat grain on the reproduction and feeding behaviour in *Rhyzopertha dominica* (F) were investigated. The evenness of distribution amongst individual wheat grains during grain treatment was not important in ensuring its effectiveness as an anti-feedant or as an insect growth regulator, provided that, overall, the grain was treated with an effective level of azadirachtin. The effect of azadirachtin on reproduction was not improved when the diluent application rates were increased from 0.5 ml kg^{-1} to 5 ml kg^{-1}. Treating 10% of the grain was shown to provide the same level of protection as treating 100% of the grain. The anti-feedant effect of azadirachtin on the beetle was also unaffected by unevenness of treatment, provided that 50% of the grain was treated. The fact that azadirachtin remains effective even when application to grain is uneven may be advantageous in field situations where uniform distribution of insecticides is difficult to achieve.

Keywords: uneven spray; azadirachtin; wheat grain; *Rhyzopertha dominica*; anti-feedant; reproduction

1 INTRODUCTION

In grain storage, protectants are sprayed onto moving grain (eg on a conveyor) which in practice results in only a fraction of the targeted grain mass being covered by the treatment. Subsequent loading in augers and on belts into bulk storage (eg a silo) results in a mixing of the treated grain with the untreated fraction.[1] It is, however, unlikely that all the grain is evenly covered by the treatment residue. It is thought that such constraints reduce the biological activity and anti-feedant effects of azadirachtin, an oxidised limonoid isolated from the neem tree, *Azadirachta indica* A Juss (Meliaceae). In accordance with this view, the literature indicates that sprays of neem-based insecticides should be applied in such a way as to ensure uniform and complete coverage because insects are able to differentiate between treated and untreated parts of their food.[2] The response of adult *Rhyzopertha dominica* (F) (lesser grain borer), a major, primary pest of stored grain throughout the world,[3] to grain that is unevenly treated was studied here as a basis for understanding the influence of uniformity of treatment on the efficacy of azadirachtin as a behavioural and physiological control agent (BCA/PCA).

Neem (containing azadirachtin) has relatively poor contact toxicity, and for most pests must be ingested to be effective.[2,4] Even though neem is an outstanding behavioural control agent (anti-feedant), its behavioural efficacy is far more variable in different species than its efficacy as a physiological control agent (an insect growth regulator.[2,4] The efficacy of neem insecticides, if not applied in high doses, is often less than that of synthetic, broad-spectrum pesticides with strong contact effects.[4] The apparent poor effectiveness of neem and other botanical pesticides, and the need to ensure complete coverage during treatment with anti-feedants, are two unattractive characteristics of natural insecticides. Such constraints inhibit large-scale usage of these products where a high level of freedom from insects is required.

In essence, coverage is dependent on the insecticide concentration and evenness of the treated residue on the commodity. However, with synthetic chemicals, evenness of distribution of residue in grain mass during application may not be critical. For example, application of malathion to 1 or 2% of grain gives as effective control as treating all the grain.[1] Non-uniform distribution of residues of insecticide does not reduce the biological activity provided the wheat is treated with an effective malathion concentration.[1,5]

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With a contact insecticide, the locomotory activity of adults results in the eventual pick-up of the toxic residue.[6] This is not the case for neem as it must be ingested for physiological effect. It is therefore the aim of this study to determine how uneven the azadirachtin distribution in a grain mass can be before it ceases to give effective control, and whether this effect can be overcome by increasing the azadirachtin concentration and/or diluent volume used. Neem has a second action, as a behavioural modifier, so we also evaluated the anti-feedant response of the insects to unevenness of azadirachtin distribution.

2 MATERIALS AND METHODS

2.1 General methods

The homozygous multi-resistant (QRD63) strain of *R dominica* used in our experiments was obtained from Queensland Department of Primary Industries (QDPI) laboratory cultures. It is a long-established laboratory reference line which has been continuously selected with fenitrothion at $12\,\text{mg}\,\text{kg}^{-1}$ in wheat.[5] The resistance factors in this non-specific malathion-resistant strain were 78×, 7.3×, and 1.7× against malathion, fenitrothion and bioresmethrin, respectively in 1990[5] (no more recent evaluation of resistance is available). Bioresmethrin is at present used in Australia specifically for the control of *R dominica*. The efficacy of current treatments with bioresmethrin is therefore threatened by the development of further resistance. Wheat used for insect culture and bioassays was organically grown (thus presumed pesticide-free) Australian standard soft wheat.

The dry neem concentrate used, donated by Dr MJ Rice, was prepared as an ethanolic extract of neem kernels (ENKE). The formulation was enriched to $5\,\text{g}\,\text{litre}^{-1}$ azadirachtin content in the solution. Azadirachtin represented 5% of the total triterpenes present in the enriched neem extract. Azadirachtin contents were measured by high-performance liquid chromotography (HPLC).[7] Dilutions were made by dissolving the ENKE in absolute ethanol. Calculation of treatment concentrations was based on azadirachtin concentration in the ENKE.

2.2 Effect on adult reproduction

We evaluated effects of grain treatment on suppression of new-generation adults by varying the application rate and fractional treatment of the grain. Grain for each test dose was treated in 100-g lots in 250-ml jars by pipetting diluted ethanolic extract onto the glass immediately above the grain surface (four jars = replicates for each test dose; grain treatment and diluent volumes used as detailed below). The jar was manually tumbled for 3 min and left overnight. In bioassays with insects, 50 one- to three-week-old adults, with the sex ratio assumed to be unity (normal for the species),[5] were added to each jar and stored in a controlled environment room at 28 °C, 55% RH, and 12:12 L:D photoperiod. Ventilation was via a filter paper lid on each jar. After 26 days of exposure and egg-laying, adults were discarded. The F1 generation was counted 10 weeks after set-up. The significance of the tested parameters, based on ANOVA of F1 emergence, was determined by Duncan Multiple Range test at $P = 0.05$.[8] Data were analysed on the basis of the effect and interaction of various parameters on insect reproduction.

2.2.1 *Effect of diluent volume and azadirachtin concentration*

The effect of uneven coverage was investigated by varying grain treatment procedure (application rates). Azadirachtin at either 1.0 or $5.0\,\text{mg}\,\text{kg}^{-1}$ was applied at diluent rates of 0.1, 0.5, 1, and $5\,\text{ml}\,\text{kg}^{-1}$ grain. The diluent application rates used were presumed to result in variation in azadirachtin deposits levels in different areas of the grain mass for the specified concentration of azadirachtin applied. For controls, four jars (=replicates) were prepared by treating wheat with ethanol only at one replicate for each application rate. Only solvent controls were used since preliminary investigations on biological activity of the ENKE showed there was no different in insect reproduction as compared to untreated control.

2.2.2 *Effect of uneven distribution of azadirachtin on grain*

The effect of uneven distribution of azadirachtin by fractionalised treatment of the grain was based on a modification of a method for testing malathion from the literature.[1] A fraction of the targeted grain mass was treated and then mixed with the untreated fraction, simulating grain treatment under field conditions. Treated grain was mixed with untreated grain in 100-g masses to produce a treated fraction of either 10, 25, 50, 75, or 100% (by weight) for each azadirachtin concentration (2.5, 5 or $10\,\text{mg}\,\text{kg}^{-1}$). Application of the diluent onto the grain was as in the previous experiment. For each replicate, a calculated concentration of azadirachtin in 1 ml was applied to the weighed fraction to be treated in a 250-ml jar. The mixture was shaken for 3 min, left overnight, following which the appropriate weighed quantity of untreated fraction was added and mixed by shaking for 20 s to give a uniform distribution. The control consisted of four wheat jars each treated with 1 ml ethanol.

2.3 Anti-feedant behaviour with unevenly treated grain

We assessed the settling behaviour of insects in choice assays because insects settle before feeding. Abraded kibbled wheat, with germ and starchy endosperm intact, was used. For each azadirachtin concentration the kibbled wheat was soaked in ENKE solution for 20 s and left to air-dry overnight. Control kernels were similarly prepared by soaking in ethanol only. Observation of anti-feedant behaviour was conducted in a test arena consisting of a 4-cm diameter glass ring placed over a 5.5-cm diameter filter paper disc (Whatman No 1) in a 9-cm glass dish. The kernels

were randomly stuck in a circle to filter paper with 'Blue-tack' (Bostik (Australia) Pte Ltd). The latter was shown to have no effect on insects in electroantennogram (EAG) studies conducted in our laboratory. Adult beetles of less than one week old were released into the centre of the arena. Assessments were based on the number of beetles that settled on the kernels after 7, 24, and 120 h. The adult settling behaviour on kernels in a given treatment at each time-point was assessed in terms of an index of infestation for each Petri dish at each assessment time. A holistic infestation index based on the number of insects on all grain in the dish, out of the total insects, was calculated as: infestation index = (no of insects feeding on treated + no on untreated kernels) ÷ total number of insects in arena. A lower value indicates greater anti-feedant effect. To normalise the variance, a logarithmic transformation [log $(x+1)$, where x = response (number of progeny)] was performed for each response. Transformed values were subjected to ANOVA, as described in Section 2.2, to compare the anti-feedant behaviour between treatments at each time-point.

The effect of uneven distribution of azadirachtin-treated kernels on insect behaviour was tested in two separate experiments, each with the following treatment combinations:

2.3.1 *One azadirachtin concentration at different ratios of treated: control kernels*

Treated kernels (50 mg kg^{-1}) were mixed with solvent control kernels at nominated ratios by the number of kernels (0:10, 1:9, 3:7, 5:5, 8:2, 10:0). Ten arenas (=replicates) were prepared for each treatment ratio. Ten beetles were released in the arena each containing ten kernels.

2.3.2 *Single-dose and multi-dose choice assays*

Each test involved 12 adults and 12 kernels. In the two-choice assays, half of the kernels were control, and the other half treated at either 10, 25, or 50 mg kg^{-1}. In multiple choice assays two different treatment combinations were tested: a mixture of 0 (solvent control), 10, 25, 50 mg kg^{-1} with three kernels from each; or a combination of 10, 25, and 50 mg kg^{-1} (ie without control) at four kernels per azadirachtin concentration. Ten replicates were prepared for each treatment.

3 RESULTS AND DISCUSSION

It is apparent from Table 1 that the activity of neem extract needed to suppress reproduction is unaffected by the diluent application rate, provided that an effective azadirachtin concentration is applied. Effective insect control appears to be more dependent on the level of azadirachtin used rather than on diluent rate, as evident from the consistently low numbers of adults obtained at an effective azadirachtin concentration (5 mg kg^{-1}); at all diluent rates (0.5–5 ml kg^{-1}) suppression exceeded 99% of the control. The mean

Table 1. Effects of application rates on the number of F1 progeny produced by *Rhygopertha dominica* in wheat treated with azadirachtin at two different concentrations

Diluent volume applied (ml ethanol kg^{-1} grain)	*Mean no adult F1 progeny (±SEM)*[a] *Azadirachtin concentration (mg kg^{-1})* 1	5
0.1	494.7 (±134.3)bA	154.0 (±10.8)bB
0.5	170.2 (±27.7)cA	11.0 (±1.2)cB
1.0	161.2 (±6.3)cA	8.7 (±0.7)cB
5.0	86.2 (±20.0)cA	2.5 (±0.2)dB

[a] Means within a column followed by the same lower case letter or means within a row followed by the same upper case letter are not significantly different ($P < 0.05$); Duncan Multiple range test. $n = 4$. Average reproduction in solvent control grain 600 (±12.1).

number of F1 progeny produced from insects exposed to solvent control was 600 (±12.1). Nevertheless, statistical analysis indicates that the biological activity of azadirachtin depends on both the applied rate of the diluent and the azadirachtin concentration. Interaction between the two parameters was significant ($n = 4$, $f = 30.4$; $P < 0.05$). At diluent rate 0.5 ml kg^{-1} or more, suppression of reproduction improves with better coverage of the residue on the grain, with significantly ($P < 0.05$) fewer F1 emergents at the highest level tested (5 ml kg^{-1}). Azadirachtin at 5 mg kg^{-1} was consistently the more effective of the two concentrations ($P < 0.05$), but even at this level it did not achieve complete control. It is conceivable that, in practical grain storage conditions, a higher diluent rate (eg 5 ml kg^{-1} or more) would not improve the effectiveness of azadirachtin. Earlier studies have shown that when ENKE (as used in this investigation) was applied at much higher rates (azadirachtin 10–50 mg kg^{-1}), complete control of F1 and F2 generations was not achieved, even though >95% reduction was obtained throughout the 48-week storage period.[7] Results in this experiment indicate that the effective application rate of 0.5 ml kg^{-1}, which is lower than commonly used either in laboratory field trials to test insecticides and insect growth regulators (1.0 ml kg^{-1}),[5] is adequate for assaying the efficacy of azadirachtin.

Data in Table 2 indicate that, at effective azadirachtin levels, treating 10% of the grain provided the same level of protection as treating 100% of the grain. At effective azadirachtin levels of 5 or 10 mg kg^{-1}, the percentage of treated grain (10% and above) tended to have no effect on the number of F1. The finding here on the effect of azadirachtin on insect control and the effects of the proportion of grain treated was similar to the previous finding on effective delivery rate of the diluent to the grain. In this experiment, interaction between the two tested parameters was significant ($n = 12$; $f = 3.09$; $P < 0.05$). The significant effect of uneven grain treatment was mainly due to the inclusion of the ineffective azadirachtin level, 2.5 mg kg^{-1}, and the influence of abnormally high F1 in one of the four replicates in the 25% grain fraction at that

Table 2. Effect on *Rhygopertha dominica* F1 progeny production of uneven distribution of azadirachtin in wheat

Grain treated (%)	Mean no adult F1 progeny (±SEM)[a] Azadirachtin concentration (mg kg^{-1})		
	2.5	5.0	10.0
10	27.3 (±12.0)aA	10.0 (±1.7)aA	28.6 (±11.6)aA
25	107.0 (±80.2)bA	6.0 (±2.2)aB	6.6 (±1.7)bB
50	13.6 (±2.1)aA	9.0a (±2.4)aA	7.6 (±1.9)bA
75	16.0 (±1.0)aA	4.6 (±0.2)aB	6.0 (±0.9)bB
100	2.3 (±1.0)cA	3.6 (±0.4)aA	4.0 (±1.2)bA

[a] Means within column followed by the same lower case letter or means within a row followed by the same upper case letter are not significantly different ($P<0.05$); Duncan Multiple range test. Average reproduction in solvent control grain [540 (±102.0)] was significant compared to all treated grain. $n=4$.

azadirachtin level (F1 was 388 compared to 27 (±7.0) for three other replicates). The high level of reproduction in this replicate was presumably due to accidental inclusion of a higher number of parents than in other replicates. The F1 progeny produced in the solvent control averaged 540 (±10), which was significantly higher than all azadirachtin treatments ($P<0.05$). The finding here shows that at effective azadirachtin levels, the strength of its action has minimised the influence of the uneven grain treatment.

Figure 1 compares varying scenarios of settling behaviour of adults at different time-points in treatments. This experiment was performed to assess varying combinations of untreated and treated kernels at one or more azadirachtin rates that are low but effective for the suppression of new generation adults. Under sustained exposure to a mixture of treated and untreated grain, the adult *R dominica* show significant anti-feedant activity when 50% or more of the grain was treated. At a fixed azadirachtin concentration (50 mg kg^{-1}) observed at intervals for five days, initially (5 and 24 h) there was a significant anti-feedant effect observed in treatments with a high percentage of treated grain (80–100%) but later (120 h) this extended to lower azadirachtin coverage (50%) (Fig 1A). The anti-feedant effect shown by treatments with 50–100% grain treated was significantly different ($P<0.05$) from those with lower percentage of grain treated.

When the effects of unevenness of azadirachtin distribution at equal proportions of treated and untreated grain were studied by varying the azadirachtin concentrations, the result showed that the anti-feedant effect was not dose-dependent (Fig 1B). The infestation behaviours in these 50% mixtures of untreated versus treated trials at azadirachtin 10, 25, 50 mg kg^{-1} showed similar levels of anti-feedant activity. When compared to different scenarios where all kernels within the dish contained 100% treated kernels of mixed concentrations (10, 25, 50 mg kg^{-1}) or when treated kernels comprised 75% of the total available kernels (0, 10, 25, 50 mg kg^{-1}) the anti-

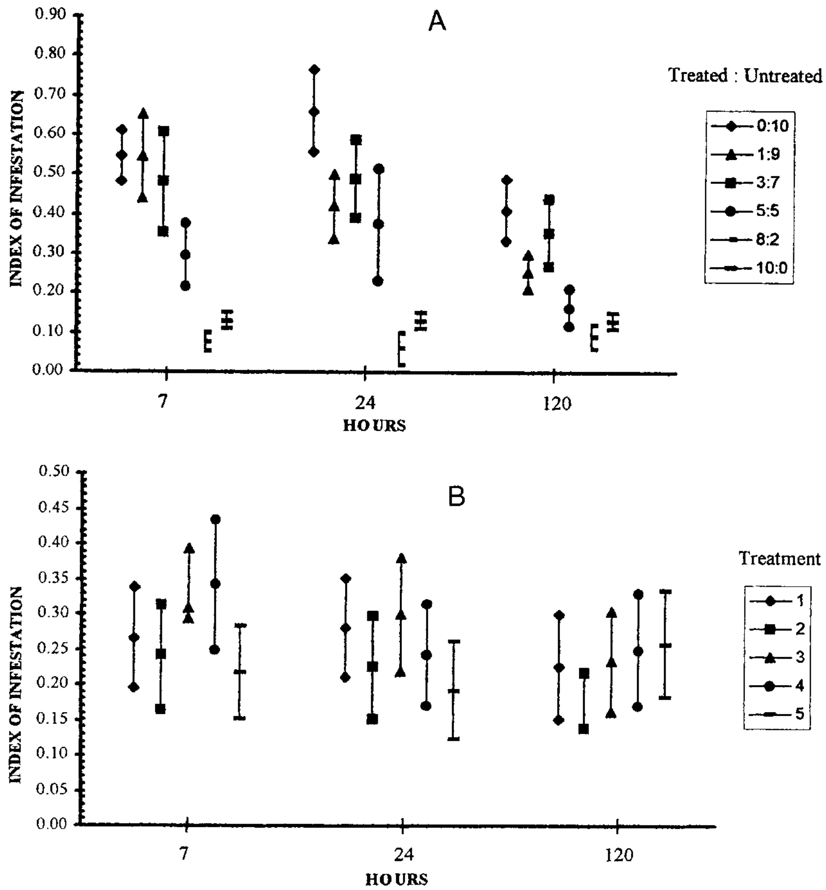

Figure 1. Settling behaviour of *Rhygopertha dominica* adults at different untreated and azadirachtin treated kernel combinations: A, nominated treated (50 mg kg^{-1}): untreated ratios; B, two-choice where treatment fraction treated at (1) 10 mg kg^{-1}; (2) 25 mg kg^{-1}; (3) 50 mg kg^{-1}; (4) multichoice of 10-25-50-mg kg^{-1}; (5) 0-10-25-50 mg kg^{-1}. Infestation index calculated as: (no. insects feeding on treated + no. on untreated kernels) ÷ total insects in arena. Ten replicates per treatment. Vertical intervals indicate standard error of mean.

feedant effect in these two and three other treatments (50% treated at a single azadirachtin concentration) was comparable at all time-points ($P < 0.05$).

Response of the adult *R dominica* to grain that is unevenly treated was studied here to understand the influence of uniformity of grain treatment on the efficacy of azadirachtin as a behavioural and physiological control agent. It was thought that uneven coverage would reduce the biological activity and anti-feedant effects of azadirachtin. This is not the case. Our behavioural assays show the relatively low significance of uniform coverage of grain on the anti-feedant behaviour of azadirachtin. When applied unevenly, azadirachtin is effective both as an anti-feedant and as a suppressant of new-generation adults.

A study on malathion applied unevenly to wheat kernels, using three stored-product beetles, including *R dominica*, showed few F1 progeny developing in any treated fraction (0.1 to 100%) for all species, even though applying the insecticide in high concentrations to a small percentage of grain (1 or 2%) was as effective as uniform treatment of all grain.[1] It has been suggested that there is a possibility of adult beetles ovipositing in a non-toxic kernel before dying, when malathion is applied unevenly to a grain mass treated at an effective concentration.[9] Behavioural assays in the present study show the importance of the feeding behaviour of *R dominica* when continuously exposed to the adverse environment of treated grain. The finding indicates that it is not important to achieve even distribution during application for azadirachtin to exert anti-feedant effects, which, according to the literature,[10] include avoidance, wandering habit, food finding, feeding and oviposition of the affected insect. In this investigation, visual observations on settling behaviour show that intially (5 h), time was spent exploring the surface of the grain as characterised by active movement in and out of the kernels. This was followed (24 and 120 h) by considerable less-active locomotion that was confined to feeding and intermittent resting (on the arena) between feeding. The intermittent feeding behaviour could be best seen in the experiment that tested mixtures of treated ($50\,mg\,kg^{-1}$) and untreated at nominated ratios at 120 h. At that time-point, an average of 5.3 beetles were observed settling on 3.9 kernels in control arenas. When compared to treatments that contained 50–100% treated kernels, the infestations in control and treated fractions were comparable (0.4–0.9 beetles settling on 0.4–0.9 kernel in control, and 0.4–2.0 beetles on 0.5–1.3 kernels in treated). These observations also suggest avoidance behaviour (primary anti-feedancy) in unevenly-treated grain. Studies on feeding behaviour show that adult *R dominica* ingest grain treated with azadirachtin when applied at the concentrations effective in suppressing reproductive activity.[11] In this study, the demonstration that treating a fraction of the grain provided the same level of protection (anti-feedant and growth regulatory[2,4]) as treating 100% of the grain suggests that incomplete reproductive control is due not only to behavioural factors, but also to the ability of the insect to survive the physiological action of azadirachtin. The low significance of uniform coverage of azadirachtin in affecting the behaviour and physiology of this insect is advantageous as compared to certain other) insecticides. This is due to the fact that one of the mechanisms for the development of biochemical resistance to the latter has long been associated with insect behavioural changes such as a positive avoidance of the treated surface.[10]

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